DYNAMICS AND MECHANISM OF IMMUNITY REACTIONS IN VIVO

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Whenever immune serum is administered for prophylactic or therapeutic purposes, pathogenic agent and antibody meet somewhere in the animal body and the reaction between the two components is appropriately described as an immunity reaction *in vivo*. These reactions are very different from the reactions with which the immunologist is usually concerned. In standardization work especially, the pathogenic agent and the antiserum are mixed *in vitro* before being injected into the experimental animal. The entire reaction takes place in the test tube and the experimental animal serves only as an indicator for the unneutralized pathogenic agent. Despite the use of experimental animals, these reactions are in reality immunity reactions *in vitro*. Thus far, immunology has been essentially a science of immunity reactions *in vitro*. Relatively little work has been done on immunity reactions *in vivo*. The present review is concerned with this particular aspect of immunology. The author and his collaborators have been concerned with this subject for some years. The interest was essentially a practical one. It was hoped that a more intimate knowledge of immunity reactions *in vivo* would furnish, or at least contribute to, a much needed theoretical foundation in serotherapy and seroprophylaxis.

Our experimental procedure was developed from this point of view. In clinical medicine one speaks of serum therapy if the serum is administered at a time when symptoms of systemic disease are already present. It appeared logical to imitate these conditions by producing the disease in the experimental animal and administering the serum as soon as the animal became sick. Experience has shown however, that it is hardly possible to work along these lines under reproducible conditions. Several authors (6, 7, 26, 49) have injected the serum at various intervals after the toxin, but before the appearance of clinical symptoms. This method has led to important results, but it has the disadvantage that nothing is known of the fate of the toxin at the time of the administration of the antitoxin.

The appearance of symptoms of a systemic disease is a certain sign that the pathogenic agent has reached susceptible tissues. As will be shown in the following sections, it is not so much the presence of symptoms as the presence of the pathogenic agent in susceptible tissues that is of paramount importance for the antiserum requirements. To work under well defined experimental conditions and to imitate at the same time the therapeutic use of antiserum as closely as possible, we injected the pathogenic agent directly into some susceptible tissue (skin, brain, or muscle) while the serum was given intravenously. Usually a constant dose of the pathogenic agent and serial dilutions of serum were given. The serum was injected immediately before the pathogenic agent. Experiments of this type will be designated as *indirect tests* and the minimal neutralizing amounts of immune serum in these tests as *Ai*.

The neutralizing dose of antiserum *in vitro* was determined with the aid of the *direct test.* In this test a constant dose of pathogenic agent and serial dilutions of antiserum were mixed and 0.1 ml of each mixture injected into the same tissue as in the indirect test. The minimal neutralizing amount of immune serum in the direct test will be designated as *Ad.*

Actual interest lies in the ratio *Ai/Ad*, i.e., the ratio between the neutralizing dose *in vivo* and the neutralizing dose *in vitro*; and since *Ai* is closely related to the therapeutic dose, one could also say that the ratio is that of the therapeutic dose to the neutralizing dose of antiserum. This ratio is independent of the potency of the immune serum and of the combining power of the test dose of the pathogenic agent. It is exclusively determined by the mechanism of immunity reactions *in vivo.* With the aid of appropriate experiments, therefore, this mechanism can be deduced from the *Ai/Ad* ratio. In the experiments to be discussed this ratio was determined for a variety of pathogenic agents and submitted to further experimental analysis.

TABLE 1

Determination of the Ai, Ad and Ai/Ad values in skin tests with diphtheria toxin

The *Ai* and *Ad* values were determined for 3 different test doses of diphtheria toxin. In three parallel experiments serial dilutions of antitoxin were injected intravenously in white rabbits weighing 2500 g. Immediately afterwards 0.1 ml. of diphtheria toxin was injected intracutaneously, in the first series in a dilution 1/10,000, in the second in a dilution 1/2,000 and in the third in a dilution 1/50. The results read after 48 hours were:

TEST DOSE OF TOXIN (0.1 ML)	DOSE OF ANTITOXIN		
	Ai (1 ml)	*Ad* (0.1 ml)	*Ai/Ad*
1/10,000	1/200	1/400,000	20,000
1/2,000	1/50	1/80,000	16,000
1/50	1/1	1/3,000	30,000

The Ai/Ad ratio and the distribution of antibody between blood and tissues

A well known example of a local reaction is the skin test with diphtheria toxin. In table 1 is recorded, in abbreviated form, an experiment in which the *Ai/Ad* ratio was determined (13) for diphtheria toxin in rabbits. Table 1 shows that, within the limits of experimental error, the *Ai/Ad* ratio is independent of the test dose of the toxin and has a value of approximately 22,000 in the rabbit. With certain variations, this figure probably holds true for other tissues, such as the heart and the adrenals. It can be concluded, therefore, that the therapeutic dose of diphtheria antitoxin will be at least 20,000 times higher than the neutralizing dose.

The high value for the *Ai/Ad* ratio becomes understandable when it is considered that only a tiny fraction of the intravenously injected antitoxin reacts with the toxin in the skin. It is very likely, therefore, that the reaction between toxin and antitoxin is determined by the relative concentrations of the two components in the tissue and not by the absolute amounts. The two experiments (13), recorded in tables 2 and 3, show the correctness of this assumption.

It will be seen from table 2 that the endpoint of titration is the same in both series, although 10 times more toxin was employed in the first series (column 2) than in the second (column 3). This experiment shows clearly that the outcome of the skin test is exclusively determined by the concentration of the toxin in the skin and is independent of the absolute amount.

TABLE 2

Dependence of the result of the skin test on the concentration of the toxin in the skin

1 ml of 1/50 diphtheria antitoxin was injected intravenously into a white rabbit weighing 2500 g. Immediately afterward serial dilutions of diphtheria toxin were injected intracutaneously, in one series in a volume of 0.1 ml and in the other in a volume of 0.01 ml.

TOXIN DILUTION	0.1 ML TOXIN RESULTS*	0.01 ML TOXIN RESULTS*
1/100	E++	E++
1/200	E++	E++
1/400	E++	E+
1/800	0	0
1/1600	0	0
1/3200	0	0

* E = erythema; 0 to ++++ indicates degree of erythema.

TABLE 3

Dependence of the result of the skin test on the concentration of the antitoxin in the skin

A 1 ml dose of diphtheria antitoxin, diluted 1/20, was injected intravenously into a rabbit weighing 2500 g, and also into a guinea pig weighing 500 g. Immediately afterwards skin tests with diphtheria toxin were performed.

TOXIN DILUTION (0.1 ML)	OUTCOME IN RABBIT*	OUTCOME IN GUINEA PIG*
1/50,000	0	—
1/20,000	0	—
1/10,000	0	—
1/5,000	0	0
1/2,000	E++++ N+	0
1/1,000	E++++ N+	0
1/500	E++++ N+	N+
1/200	—	N+++
1/100	—	N+++

* E = erythema; N = necrosis; 0 = no reaction; — = not done.

Table 3 shows that, for the same dose of antitoxin, the maximal amount of neutralized toxin is inversely proportional to the body weights of the experimental animals. This result strongly suggests that the outcome of the skin test depends on the concentration of the antitoxin in the blood plasma and is, therefore, determined by its concentration in the skin.

There is little doubt that the high *Ai/Ad* ratio, in the skin test with diphtheria toxin, is due chiefly to the dilution of the antitoxin in the blood plasma and to its low concentration in the skin tissue. This also seems to follow from the fact that the same *Ai/Ad* ratio of 22,000 was found (13) in experiments with staphy-

lococcus toxin. It is of great interest to calculate the influence of these two factors on the Ai/Ad ratio. Assuming that other factors can be neglected, the minimal neutralizing concentrations of antitoxin in the skin will be identical in the direct and the indirect tests. This concentration is Ad/V in the direct test and Ai/PK in the indirect test.

$$\text{Hence, } Ai/Ad = PK/V, \text{ or } K = AiV/AdP \qquad (a)$$

In these formulas V represents the volume of intracutaneously injected fluid, P the plasma volume, and K the coefficient of the distribution of antitoxin between plasma and skin. Were the ratio exclusively determined by these factors, it should obviously have the same value for all pathogenic agents. Actually immunity reactions *in vivo* may be and often are of a much more complicated character. It will be seen later that the tissue cells may take part in several ways in the reaction between pathogenic agent and antibody and thereby control the Ai/Ad ratio. In this case equation a does not hold true. Nevertheless, the equation is not altogether worthless. It indicates what the Ai/Ad ratio would be if it were exclusively determined by the dilution of the antitoxin in the blood plasma and its low concentration in the skin, irrespective of the question whether this condition is ever realized.

It is a fortunate circumstance that the skin test with diphtheria toxin furnishes the material needed for the application of the equation. It has been shown (12) that the reaction between diphtheria toxin and antitoxin is extremely rapid. In the skin, the reaction is complete before the toxin has opportunity to react extensively with the tissue. Therefore, the velocity of the reaction between toxin and tissue is without importance for the Ai/Ad ratio and equation a is valid.[1] This is proved independently by the following consideration. K can be calculated from the equation if Ai and Ad have been determined experimentally. If $Ai/Ad = 22{,}000$, $P = 100$ and $V = 0.1$, then K has the value 22, i.e., the concentration of antitoxin in the skin is approximately 5% of that in the blood plasma. Since antibodies (in this case, diphtheria antibody) are either globulins or closely linked to them, K should have the same value for antitoxins and globulins. This is actually the case. According to Peters (44), the globulin content of the tissue fluid is approximately 5% of that of the blood plasma.[2]

With a view to later experiments with neurotropic viruses, the Ai/Ad ratio was determined for diphtheria toxin in intracerebral tests in guinea pigs weighing 250 g (14). The value found was 6200. If the body weights of the two species (rabbit and guinea pig) are taken into consideration, it is apparent that the

[1] The lengthy but conclusive proof of this statement would interrupt the flow of presentation. The interested reader may be referred, therefore, to the original paper (12).

[2] This result meets a criticism (53) that has been levelled against our method of calculation. It has been claimed that diphtheria toxin is a capillary poison and that, consequently, the method cannot possibly measure the normal permeability of the capillaries. In this case, however, K could not have the same value for antitoxins and globulins. The reason for the validity of the equation, in this case, is the long incubation period of diphtheria toxin. In the skin test it takes many hours before any effect of the toxin on the capillaries becomes visible in the form of erythema. Long before that can happen, the toxin is neutralized by the antitoxin (12).

Ai/Ad ratio for the brain is higher than for the skin. That is as it should be, for according to Freund (9) the antibody concentration in the brain is lower than in most other tissues.

The value 6200 is of great importance. It shows what the *Ai/Ad* ratio would be for neurotropic viruses if it were determined simply by the distribution of the antibody between blood and brain. It will be seen in a subsequent section that the deviation of the experimentally determined *Ai/Ad* ratio from the figure 6200 gives valuable information concerning the mechanism of immunity in neurotropic virus diseases.

This may be the opportunity to mention some earlier investigations although they are only loosely connected with the subject. It was early recognized that the therapeutic dose of antitoxin was infinitely greater than the neutralizing dose and several attempts were made to explain this difference. All these investigations have in common the fact that the antitoxin was injected at various intervals after the toxin.

Doenitz (6, 7) injected 15 MLD of diphtheria toxin intravenously into rabbits, forty minutes later even 11,000 times the amount of antitoxin which neutralized the test dose of toxin *in vitro* was unable to protect the animals. Schick *et al.* (49) determined the amount of intramuscularly injected antitoxin which prevented a positive Schick test in children. The Schick reaction failed to appear when the antitoxin was injected simultaneously with the toxin, but was positive when antitoxin was injected 3 or 6 hours after the toxin.

Glenny and Hopkins (26) injected diphtheria toxin intracutaneously into rabbits and, after various intervals, antitoxin was injected intravenously. Fifteen minutes after the intracutaneous injection of the toxin, 10,000 times the neutralizing dose of antitoxin was unable to prevent a positive skin reaction. After thirty minutes even one million times the neutralizing dose was ineffective.

From these experiments it has been concluded that the combination between toxin and tissue undergoes a gradual increase in firmness and that, consequently, it becomes increasingly difficult for the antitoxin to dislodge the toxin from the cells. I think it is still the general opinion that this reaction between the toxin and the tissue is the only reason for the difference between the neutralizing and the therapeutic dose of antitoxin. The experiments reported in the first part of this section show that this is not the case. The most important factor is the separation of the toxin in the tissues from the antitoxin in the blood by the capillary wall. It is very likely, also, that this factor plays an important role in the interpretation of the experiments of Doenitz and of Glenny and Hopkins. Nevertheless, there is no doubt that the difference between the therapeutic and the neutralizing dose of antitoxin is at least partly due to the reaction between toxin and tissue. The *Ai* value, therefore, differs from the therapeutic dose by a factor which takes account of this reaction.

The systemic action of diphtheria toxin and the Ai/Ad ratio. Pathogenesis of tetanus

In the preceding section the skin test with diphtheria toxin was discussed as an example of the local action of the toxin on the tissue into which it had been

injected. In the present section the action of the toxin on organs remote from the site of its injection will be described. It will soon be seen that this experiment has some important implications.

A representative experiment is recorded in table 4 (21). As may be seen from this table, intramuscularly injected toxin does not require even twice as much antitoxin as intravenously injected toxin. The antitoxin requirements would probably be exactly the same were it not for the effect of the Danysz phenomenon. The toxin is slowly absorbed from the muscle, and therefore, requires more antitoxin than the same dose of toxin injected intravenously. Accordingly we have found in unpublished experiments that intravenously injected toxin required for its neutralization the same amount of antitoxin as intramuscularly injected toxin, if the former was injected in four portions separated from each other by intervals of two hours. The explanation of these experiments is simple.

TABLE 4

Antitoxin requirements for intramuscularly and intravenously injected diphtheria toxin

Serial dilutions of diphtheria antitoxin were injected intravenously into guinea pigs weighing 250 g. Immediately afterward, 20 lethal doses of diphtheria toxin were injected intramuscularly in series A, and intravenously in series B. Death or survival of the experimental animals was recorded.

ANTITOXIN (0.5 ML)	SERIES A*	SERIES B*
1/100	S,S,S,S	S,S
1/200	S,S,S,S	S,S
1/400	S,5,6,5	S,S
1/800	2,2,2,2	S,1,2,3,5

* S = survived; numerals indicate day of death.

In order to protect the animals in these experiments, it is necessary to neutralize the toxin before it reaches the heart or the adrenals, but it is immaterial whether it is neutralized at the site of its injection or in the blood. Theoretically, therefore, intravenously and intramuscularly injected toxin should be neutralized by the same amount of antitoxin.

It is interesting to compare the result of the indirect intramuscular test with that of the indirect intracutaneous test. To do this it will be convenient to define the direct test in a more general way. As before, the term "indirect test" will be the designation for experiments in which toxin and antitoxin are injected into separate compartments. The term "direct test", however, will now include all experiments in which toxin and antitoxin are injected into the same compartment, e.g., the vascular system. The minimal neutralizing amount of antitoxin in such an experiment will again be designated as Ad .The above results can now be formulated as follows. In the skin test, in which the local action of the toxin was considered, the Ai/Ad ratio was 22,000. In the intramuscular test, in which the systemic action of the toxin was dealt with, the Ai/Ad ratio approached unity. The reason for this is the fact that in the skin test, in order to obtain the

result, it is necessary that the toxin be neutralized at the site of its injection. This, however, is immaterial in the intramuscular test.

These rather obvious considerations found an unexpected and important application in the case of tetanus toxin. If this toxin reached the central nervous system by way of the circulation, the *Ai/Ad* ratio in the indirect intramuscular test should be the same as with diphtheria toxin, i.e., unity. In this case it would be immaterial whether the toxin is neutralized at the site of its injection or in the vascular system. If, on the other hand, according to Meyer and Ransom (38) the toxin reaches the central nervous system by way of the peripheral nerves, the animals can be protected only if the toxin is neutralized in the muscle. This neutralization, in turn, depends on the concentration of the antitoxin in the muscle tissue. Therefore, the *Ai* value and consequently, the *Ai/Ad* ratio must be high. By determining this ratio it should be possible, therefore, to decide

TABLE 5

Antitoxin requirements of intramuscularly and intravenously injected tetanus toxin

In two series of experiments, serial dilutions of tetanus antitoxin were given intravenously to guinea pigs weighing 250 g. Immediately afterwards 20 lethal doses of tetanus toxin (Lederle) were injected intramuscularly in one series and intravenously in the other.

ANTITOXIN (0.5 ML)	TOXIN INTRAMUSCULARLY, RESULTS*	TOXIN INTRAVENOUSLY, RESULTS*
1/20	L.T.	—
1/40	L.T.	—
1/100	5	—
1/200	5	—
1/400	5	—
1/800	3	S
1/1600	—	S
1/3200	—	S
1/6400	—	3

* L.T. = local tetanus; S = survived; — = not done; numerals indicate day of death.

whether tetanus toxin reaches the central nervous system by way of the circulation or by way of the peripheral nerves. The experiment recorded in table 5 gives a clearcut answer to this question (16). As may be seen, the *Ai/Ad* ratio in the indirect intramuscular test as defined above, is 80:1, whereas it was near to unity in the case of diphtheria toxin.

Further experiments have shown very clearly that the high *Ai/Ad* ratio for tetanus toxin can be explained only by the migration of the toxin in the peripheral nerves. It was found that after sectioning of the sciatic and femoral nerves the *Ai/Ad* ratio attained the same value as for diphtheria toxin, namely, unity (17). This experiment is demonstrated in table 6.

The following experiments show that in accordance with the theory of Meyer and Ransom only sectioning of the motor nerve roots has this effect; whereas sectioning of the posterior nerve roots has no influence on the *Ai/Ad* ratio (18). These experiments were carried out in rhesus monkeys. In preliminary tests

it was determined how much intravenously injected antitoxin was necessary to protect the monkeys against 25 MLD of tetanus toxin given either by the intramuscular or intravenous route; 1 ml of a 1/3200 dilution of antitoxin protected against the intravenously injected toxin, whereas the intramuscularly injected toxin required 1 ml of a 1/100 dilution. In the main experiment, 1 ml of a 1/600 dilution of antitoxin was given. The intramuscularly injected control animals died within a few days. Four animals in which the posterior nerve roots had been transsected prior to the experiment, died at the same time as the controls. Three other monkeys whose anterior nerve roots had been cut survived the injection of a dose of toxin which killed the controls and the animals with the

TABLE 6

Antitoxin requirements for intramuscularly and intravenously injected toxin and for intramuscularly injected toxin after denervation of the leg

Serial dilutions of tetanus antitoxin were given intravenously to guinea pigs weighing 250 g. In series I, 20 lethal doses of tetanus toxin (Lilly) were given intramuscularly; in series II, intravenously. In series III, the femoral and sciatic nerves were cut before the intramuscular injection of the toxin.

ANTITOXIN (1 ML)	SERIES I: TOXIN INJECTED INTO INTACT MUSCLE RESULTS*	SERIES II: TOXIN INJECTED INTRAVENOUSLY RESULTS*	SERIES III: TOXIN INJECTED INTO DENERVATED MUSCLE RESULTS*
1/16	L.T.	S	S
1/128	L.T.	S	S
1/256	3	S	S
1/512	2	S	S
1/1,024	2	S	S
1/2,048	—	G.T.	L.T.
1/4,096	—	2	3
1/8,192	—	2	—
1/16,384	—	1	—

* L.T. = local tetanus; G.T. = generalized tetanus; S = survived; — = not done; numerals indicate day of death.

dissected posterior nerve roots. This experiment shows that the *Ai/Ad* ratio is high only when the motor nerve pathway is intact.

From the observations discussed in this section follow some important general rules concerning the *Ai/Ad* ratio.

1. The *Ai/Ad* ratio is always high when at the time of injection of the antitoxin the toxin is already in the susceptible tissue (as in the skin test with diphtheria toxin).

2. If at the time of the injection of the antitoxin, the pathogenic agent is still outside the susceptible tissue (indirect intramuscular test with diphtheria toxin) the *Ai/Ad* ratio approaches unity, provided that the pathogenic agent reaches the susceptible organ by way of the circulation.

3. Under the same circumstances the *Ai/Ad* ratio is high if the pathogenic agent reaches the susceptible organs along the nerve pathways.

Quantitative determination of capillary permeability with the aid of the indirect test (22)

In the course of the investigations, it was observed that, in the indirect skin test with diphtheria toxin, the effect of antitoxin was strongly enhanced when the toxin was dissolved in the solution of certain substances other than NaCl. This effect was very marked with nutrient broth as may be seen from table 7. Twenty-two times more toxin was neutralized in series B (solvent, broth) than in series A (saline). The result of the direct skin test was in no way affected by broth; hence the possibility that broth has a direct influence on the reaction between toxin and antitoxin was excluded.

TABLE 7

Antitoxin requirements in indirect skin tests with diphtheria toxin dissolved in saline or nutrient broth

1 ml of 1/50 diphtheria antitoxin 874 (1600 units per ml) was injected into a white rabbit weighing 2500 g. Immediately afterwards serial dilutions of diphtheria toxin 1116 in a volume of 0.1 ml were injected intracutaneously. In series A, the toxin was diluted in saline, in series B, it was diluted in nutrient broth. Results were read after 48 hours.

TOXIN DILUTIONS	SERIES A RESULTS*	SERIES B RESULTS*
1/100	E	N
1/150	—	N
1/200	E	E
1/250	—	E
1/300	—	0
1/400	E	0
1/800	E	0
1/1600	E	0
1/3000	E	—
1/4500	E	—
1/6000	E	—
1/6500	0	—
1/7000	0	—

* N = necrosis; E = erythema; 0 = no reaction; — = not done.

The result would obviously be explained if it were assumed that broth increases the permeability of the capillaries to antitoxin. There were several ways whereby the correctness of this assumption could be verified. The increased permeability to antitoxin should influence the indirect test only when the concentration of the antitoxin at the site of injection of the toxin determines the result of this test. According to the findings in the preceding section, therefore, broth should increase the effect of antitoxin in the intramuscular test with tetanus toxin but should fail to do so in the intramuscular test with diphtheria toxin. Tables 8 and 9 show that this is exactly what happened.

As may be seen from table 8, the antitoxin titer was the same in both series. In other words, broth had not altered the result of the indirect intramuscular

test with diphtheria toxin. As table 9 indicates, the animals in series B (broth) were protected by $\frac{1}{8}$ of the amount of tetanal antitoxin which was required in series A (saline). These experiments show clearly that broth enhances the effect of antitoxin in the indirect test with tetanus toxin, by increasing the permeability of the capillaries to the antitoxin.

TABLE 8

Indirect intramuscular test with diphtheria toxin dissolved in saline or nutrient broth

Serial dilutions of diphtheria antitoxin 874 (1600 units per ml) were injected intravenously in 1 ml amounts into guinea pigs weighing 250 g. Immediately afterwards, 20 lethal doses of diphtheria toxin 1116 were injected intramuscularly. In series A, the dilutions of toxin were made in saline; in series B, they were made in broth.

ANTITOXIN (1 ML)	SERIES A (SALINE) RESULTS*	SERIES B (BROTH) RESULTS*
1/200	S,S	S,S
1/400	5,6	3,3
1/800	2,2	2,2

* S = survived; numerals indicate day of death.

TABLE 9

Indirect intramuscular test with tetanus toxin dissolved in saline or nutrient broth

1 ml amounts of serial dilutions of tetanus antitoxin 387 (1200 units per ml) were injected intravenously into guinea pigs weighing 250 g. Immediately afterwards, 20 lethal doses of tetanal toxin 1556 in a volume of 0.1 ml were injected intramuscularly. In series A the toxin was diluted in saline; in series B, in broth.

ANTITOXIN (1 ML)	SERIES A (SALINE) RESULTS*	SERIES B (BROTH) RESULTS*
1/10	L.T., L.T.	—
1/20	L.T., L.T.	—
1/40	6,6	S,S
1/80	2,2	L.T., L.T.
1/160	3,3	L.T., L.T.
1/320	—	L.T., 5
1/640	—	2,2
1/1280	—	—

* L.T. = local tetanus; S = survived; — = not done; numerals indicate day of death.

The effect of broth on the capillaries could be demonstrated even more directly with the aid of aniline dyes. Five ml of a 1% solution of Evans blue was injected into a white rabbit weighing 2500 g. The depilated skin assumed a very slight blue color. The intracutaneous injection of 0.1 ml of saline produced no visible change in the skin. After the injection of 0.1 ml of broth even in a dilution of 1/30, however, the bleb stained a deep blue.

The indirect intracutaneous test with diphtheria toxin and the indirect intramuscular test with tetanus toxin have been employed in a study of the effect of a number of chemicals and biological fluids on the permeability of the capil-

laries. The results are briefly summarized in table 10. Although the effect of substances on capillary permeability can be and has been studied with the aid of physiological methods, the latter are mostly of an indirect nature and measure the effect on lymph production rather than on capillary permeability directly. Both do not necessarily run parallel. Egg albumin, for instance, is a strong lymphagogue, but in our experiments it had no effect on the capillaries of the skin. Duran-Reynals (8) found that testicular extract dilated the capillaries but left unanswered the question whether or not this effect was due to the spreading factor. Our quantitative method permitted the demonstration that the spreading factor is destroyed by boiling for a few minutes while the capillary factor in testicular extract is thermostable.

Of particular interest is the effect of the blood group substances on capillary permeability. These substances were investigated because they are present in commercial peptones. The substances with blood group properties, isolated by

TABLE 10

Effect of substances on capillary permeability

DILATE CAPILLARIES	LEAVE CAPILLARIES UNAFFECTED
Nutrient broth	Blood serum
Difco peptone	Cerebrospinal fluid
Savita broth without peptone	Egg albumin
Histamine	Liver extract
Testicular extract	Kidney extract
Leech extract	Histidine
Saliva	Arginine
Saline extract of feces	Cysteine
Blood group substances	Inositol
	Biotin

Witebsky (54) and produced commercially, as well as a blood group A substance prepared in a chemically pure form by Kabat *et al.* (31, 3), had a strong effect on the capillaries. Biological and clinical implications of this observation will not be discussed here.

As was to be expected, histamine has a very strong effect on capillary permeability. Even in a dilution of 1/100,000 this effect is still marked. We have shown that our method can be used to study the effect of antihistaminic drugs quantitatively (51).

From the physiological point of view the quantitative aspect of the problem is of particular interest. It has been shown in table 7 that broth increases the permeability of the capillaries of the skin to antitoxin about twenty-two times. It will be recalled that according to table 1, the normal coefficient, K, of the distribution of antitoxin between blood and skin was likewise twenty-two. It can easily be shown that the identity of these two figures is no coincidence. Assume that the equation for the normal capillaries reads $Ai/Ad = KP/V$. After artificial increase of the capillary permeability, the equation takes the form

$Ai'/Ad = K'P/V$. Hence, $Ai/Ai' = K/K'$. Now it is obvious that maximal permeability of the capillaries will be reached when the concentration of antitoxin in blood and tissue have become identical. In this case $K' = 1$ and $Ai/Ai' = K$.

For the theory of capillary permeability, it is a remarkable fact that substances which have no apparent destructive properties for cell structures remove all barriers between blood and tissue in a reversible manner.

The avidities of toxins for tissues and antitoxins

In a preceding section, it has been shown that in skin tests with diphtheria toxin, the Ai/Ad ratio can be defined by the equation $Ai/Ad = KP/V$, and that in this case the experimentally determined ratio is 22,000. This simple relation was shown to be due to the fact that in the case of diphtheria toxin, the Ai/Ad ratio is determined exclusively by the distribution of antitoxin between blood and tissue; and this in turn was shown to be a consequence of the very rapid reaction between toxin and antitoxin. Diphtheria toxin has no opportunity to react with the tissue in the presence of antitoxin.

It would be unjustified, however, to assume that the same simple relations hold true for all pathogenic agents. In contrast to the immunity reactions *in vitro*, the immunity reactions *in vivo* take place in the presence of susceptible tissue cells and the interaction between pathogenic agents, antibodies, and tissues may have a marked effect on the Ai/Ad ratio. This section will deal with these more complicated matters.

As is well known, the reaction between tetanus toxin and its antitoxin is much slower than the reaction between diphtheria toxin and antitoxin. The possibility must be considered, therefore, that a reaction between toxin and tissue might interfere with the reaction between toxin and antitoxin and that this interference might be reflected in the value of Ai/Ad. An attempt to approach this problem experimentally brought forth a very unexpected observation (19). It was found that the Ai/Ad ratios for individual tetanus toxins showed tremendous differences. In a summarized form, these results are recorded in table 11 which shows that the Ai/Ad ratios vary over a range from 100 to 12,500. The Ad values do not differ significantly. But the Ai values for toxins 1175H and 641B are 100 times higher than that for toxin 388. The differences in the ratios, therefore, are predominantly due to differences in the Ai values. In principle, the same observation was made in indirect intramuscular tests with tetanus toxin (21). Table 12 shows that the Ai value for toxin 641B is approximately 62 times higher than that for toxin B.

While in the direct test 20 lethal doses of all tetanus toxins are neutralized by approximately the same amounts of antitoxin, there exist qualitative differences between individual tetanus toxins which could be discovered only with the aid of the indirect test. The next problem was the experimental analysis of these differences.

In the indirect intracerebral test we are dealing with immunity reactions *in vivo*. Two reactions take place at the same time in the central nervous system,

namely a reaction between toxin and antitoxin and a reaction between toxin and nerve tissue. The result, therefore, will depend not only on the concentrations of the two components but also on the velocities of the reactions between toxin and antitoxin, and toxin and tissue, respectively. A high avidity of the toxin for tissue will increase the antitoxin requirements, while a high avidity of the toxin for antitoxin will decrease them.

TABLE 11

Ai, Ad and Ai/Ad values for 7 tetanus toxins

In the indirect test, serial dilutions of tetanus antitoxin were injected intravenously into guinea pigs weighing 250 g. Immediately afterwards, 20 MLD of the individual toxins were given intracerebrally. In the direct test, a constant dose of toxin and serial dilutions of antitoxin were mixed *in vitro*, and 0.1 ml of the mixtures containing 20 MLD were injected intracerebrally.

TOXIN SAMPLE	*Ai* (ml)	*Ad* (ml)	*Ai/Ad*
388	0.002	—	—
47	0.008	—	—
64	0.0031	0.000031	100
103	0.0062	0.000031	200
Lilly	0.04	0.000085	470
1175 H	0.2	0.000016	12,500
641 B	0.2	0.000016	12,500

TABLE 12

Indirect intramuscular tests with 9 tetanus toxins

Serial dilutions of antitoxin 387 (1200 units per ml) were given intravenously. Intramuscular test dose of each toxin was 20 MLD.

TOXIN SAMPLE	*Ai* (ml)	TOXIN SAMPLE	*Ai* (ml)
641 B	0.05	J	0.0062
1556	0.025	K	0.0031
1346	0.0125	L	0.0031
1375	0.0125	B	0.0008
H	0.0062		

Under certain experimental conditions, the velocity of the reaction between toxin and antitoxin can be measured by the velocity of flocculation in the Ramon test. Of course differences in the potencies of the toxins must be eliminated by proper dilutions. After doing this, the flocculation rates were found (21) to be approximately the same for all tetanus toxins examined. Consequently, the differences in the *Ai* values cannot be explained by differences in the avidities of the toxin for antitoxin.

The following experiments (21) will show that the differences in the *Ai* values are determined by differences in the avidities of the toxins for nerve tissue. The possibility of proving this matter experimentally is due to the fortunate

circumstance that yet another phenomenon is determined by the avidity of the toxin for nerve tissue. As mentioned in an earlier section, the antitoxin requirements increase with the interval between the injection of toxin and the subsequent injection of antitoxin. This has been explained by assuming that the combination between toxin and tissue undergoes a gradual increase in firmness and that, consequently, it becomes increasingly difficult for the antitoxin to dislodge the toxin from the tissue. If this explanation is accepted, it implies

TABLE 13

Increase of antitoxin requirements associated with the interval between injection of tetanus toxin and antitoxin

Two identical experiments were performed with toxins 64 and 641 B. In both, 20 lethal doses of toxin were injected intracerebrally. In series A, serial dilutions of antitoxin were given intracerebrally immediately after the toxin. In series B, the antitoxin was given 3 hours later.

ANTITOXIN (ML)	TOXIN 64 MLD = 0.1 ML, 1/50 TEST DOSE = 0.1 ML, 1/2.5 (20 MLD) RESULTS*		TOXIN 641B MLD = 0.1 ML, 1/1600 TEST DOSE = 0.1 ML, 1/80 (20 MLD) RESULTS*	
	A	B	A	B
0.016	—	—	—	S,S
0.008	—	—	—	S,3
0.004	—	S,S	—	S,3
0.002	—	S,S	—	2,2
0.001	—	S,7	—	1,2
0.0005	—	6,8	—	1,1
0.00025	—	4,7	—	—
0.000125	S,S,S	2,7	—	—
0.000062	S,5	2,2	S,S	—
0.000031	4,4	—	S,6	—
0.000016	2,2	—	2,7	—
0.000008	2,4	—	1,2	—
0.000004	—		1,2	—
Protecting dose (ml).....	0.000125	0.002	0.000062	0.016
B:A.....	16:1		260:1	
Ai.....	0.003		0.2 ml	

* — = not done; S = survived; numerals indicate day of death after injection.

that the increase in the antitoxin requirement should be determined by the avidity of the toxin for nerve tissue. If the *Ai* value were likewise determined by this avidity, there should be positive correlation between the *Ai* values and the increase in the antitoxin requirements associated with the interval between the injections of toxin and antitoxin.

To test the validity of these conclusions an experiment was performed with toxin 64 (*Ai* = 0.003 ml) and toxin 641B (*Ai* = 0.2 ml). It will be seen from table 13 that the increase in the antitoxin requirements owing to the interval between the injection of toxin and antitoxin is indeed correlated with the *Ai*

values. The increase was 16:1 for toxin 64 with an *Ai* value of 0.003 ml, but 260:1 for toxin 641B with an *Ai* value of 0.2 ml. Since differences in the avidities of the toxins for antitoxin were excluded by previous experiments, these results can be interpreted only as indicating that the differences in the *Ai* values are determined by the differences in the avidities of the tetanus toxins for nerve tissue.

These results illustrate the usefulness of studying immunity reactions *in vivo*. The hitherto unknown qualitative differences between individual tetanus toxins could be discovered only with the aid of the indirect test, whereas they passed unnoticed in the direct test.[3] In several respects, the results should be of some practical significance. The experiments show that tetanus toxins with high avidities for nerve tissue are practically resistant to antitoxin under conditions as they prevail in the natural disease. Our results show further that in the case of tetanus toxin, the standardization of the antitoxin is of limited value. The standardization methods are based on the assumption that the antitoxin requirements are determined by the combining power of the test dose of toxin. In the indirect test and in the natural disease, the antitoxin requirements are to a large extent dependent on the avidity of the toxin for nerve tissue. Since this avidity varies from case to case and is unknown to the clinician, there is little hope that any standardization method will be able to overcome this difficulty.

In the preceding paragraphs, it has been shown that in the case of tetanus toxin, the *Ai/Ad* ratio, and in a broader sense, the curative effect of antitoxin is strongly affected by the avidity of the toxin for nerve tissue. It was shown at the same time that individual tetanus toxins do not vary in their avidities for antitoxin. It will now be shown that various samples of antitoxin may vary in their avidities for the same toxin, and in their curative values. Earlier investigations did not make use of the method of the *Ai/Ad* ratio, but they are closely related to our subject because they deal with the problem of immunity reactions *in vivo*. Roux was the first to observe that the curative values of antidiphtheric sera did not always run parallel to their potencies expressed in terms of units of antitoxin. This question was investigated on a much larger scale by Kraus and Schwooner (34) and by Kraus and Baecher (32). They first injected toxin into guinea pigs and then antitoxin after varying intervals. In conformity with the results of Roux, they found no parallelism between the curative powers of the sera and their potency in terms of units of antitoxin. Kraus advanced the theory that the curative value of antitoxic sera is dependent not only on their strength as determined by the method of Ehrlich, but also on the avidities of the antitoxins for toxin. These experiments led to a lively but inconclusive controversy between Kraus and the Ehrlich school.

[3] After it had been found that broth strongly increases the effect of antitoxin in the indirect test, the possibility was considered that the differences in the *Ai* values of the individual toxins might be due to differences in the broth contents of the test doses. In this case, the *Ai* value should be determined by the potency of the toxins. However, in a large number of experiments no correlation was found between these two quantities. The effect of broth on capillary permeability diminishes rapidly upon dilution and this is probably the reason why it has little influence on the *Ai* value.

The question was finally settled by the experimental investigations of Madsen and Schmidt (36, 37). They observed that mixtures of diphtheria toxin and some antitoxins were neutral when injected subcutaneously into guinea pigs but were highly toxic when injected intravenously into rabbits. Other antitoxic sera did not show this phenomenon. Madsen and Schmidt concluded that sera of the first type react slowly with toxin while sera of the second type react rapidly. This explanation was supported by the observation that sera of the first type usually precipitated toxin more slowly in the Ramon test than did sera of the second type. Finally it was found that sera of the second type were therapeutically more potent than sera of the first type. These investigations, therefore, can be considered as conclusive evidence for the theory of Kraus.

Glenny and his coworkers (24, 25, 27) have tried to define the concept of avidity more precisely. They do not consider the rapidity of the reaction between toxin and antitoxin as essential. Of greater importance is the firmness of the combination between the two. The mixtures of toxin and some antitoxins dissociate readily upon dilution. For this reason a mixture of large amounts of toxin and antitoxin may be neutral while a tiny fraction of this mixture may be highly toxic. For example, Glenny and Barr (24) prepared a mixture of toxin and antitoxin which was neutral when injected intravenously into a rabbit in a volume of 10 ml, whereas, 0.001 to 0.5 ml was lethal. Glenny *et al.* have developed methods with the aid of which the avidities of antitoxins for toxin can, to some extent, be measured quantitatively. The reader may be referred to the discussion of this work by Wilson and Miles (53).

The Ai/Ad ratio and the mechanism of immunity in neurotropic virus diseases

In the field of neurotropic virus diseases, the *Ai* and *Ad* values stand for familiar concepts. The *Ad* value represents the neutralizing amount of antiserum in the customary neutralization test. The *Ai* value measures the minimal amount of antiserum which provides passive protection against the intracerebral injection of the virus. The *Ai/Ad* ratio, therefore, is the ratio between the passively protecting and the neutralizing dose of antiserum.

According to the indirect intracerebral tests with diphtheria toxin discussed earlier, the *Ai/Ad* ratio should be 6200:1 for all neurotropic viruses, if this ratio were exclusively determined by the distribution of the antibody between blood and brain. It can be shown, however, that the *Ai/Ad* ratios for some of the neurotropic viruses differ widely from the "standard" value of 6200:1, and further, that the *Ai/Ad* ratios for the individual viruses differ widely among themselves.

The *Ai/Ad* ratio for the virus of rabies was determined (20) in the experiments recorded in table 14. As may be seen, 1 ml of undiluted serum did not protect guinea pigs passively against as little as 10 lethal doses. It is impossible, therefore, to determine an *Ai/Ad* ratio. Since, however, the *Ad* value was 0.1 ml, 1/64, the ratio was at least 1240 and consequently of the same order as that for diphtheria toxin.

In the experiments recorded in table 15, the *Ai/Ad* ratio was determined for

the virus of Eastern equine encephalomyelitis (20). The results indicate that the *Ai/Ad* ratio for the virus of equine encephalomyelitis is 5:1. This extremely low value is totally at variance with the "standard" value of 6200:1 and ob-

TABLE 14

Determination of the Ai and Ad values for the virus of rabies in guinea pigs weighing 200 g

In the indirect test, serial dilutions of antiserum were given intravenously. Immediately afterwards 0.1 ml of a 1/100,000 dilution of rabies virus (10 MLD) was injected intracerebrally. In the direct test, 0.3 ml of a virus dilution 1/50,000 and 0.3 ml of serial serum dilutions were mixed *in vitro* and 0.1 ml of each mixture injected intracerebrally without incubation.

DETERMINATION OF *Ai*		DETERMINATION OF *Ad*	
Serum dilution (1 ml dose)	Survivals*	Serum dilution (0.1 ml dose)	Survivals*
Undiluted	9,9	1/4	S,S
1/2	9,9	1/8	S,S
1/4	9,9	1/16	S,S
1/8	8,8	1/32	S,S
		1/64	S,S
		1/128	9,8
		1/256	9,9

* S = survived; numerals indicate day of death.

TABLE 15

Determination of the Ai and Ad values for the virus of eastern equine encephalomyelitis

In the indirect test, serial dilutions of antiserum were given intravenously while immediately afterwards 0.1 ml of a 1/100 suspension of the virus (160 MLD) was injected intracerebrally. In the direct test, 0.3 ml of 1/50 virus dilution and 0.3 ml of serial serum dilutions were mixed *in vitro*, and 0.1 ml of each mixture injected intracerebrally. The experiments were carried out in guinea pigs weighing 200 g.

DETERMINATION OF *Ai*		DETERMINATION OF *Ad*	
Antiserum (ml)	Survivals*	Antiserum (ml)	Survivals*
0.5	S,S	0.05	S,S
0.25	S,S	0.025	6,7
0.125	6,10	0.0125	8,10
0.062	6,7	0.0062	6,6
0.031	5,10	0.0031	4,6
0.016	4,4	0.0016	4,10
Ai = 0.25 ml		*Ad* = 0.05 ml	

* S = survived; numerals indicate day of death.

viously calls for an explanation. An important step in this direction was made by the fundamental investigation of Olitsky and Harford (42). These authors found that equine encephalomyelitis antiserum has a very low potency in the direct intracerebral test but is very potent in the intraperitoneal test in young

mice. In confirmation of these results we have found that 0.1 ml of a 1/250 dilution of serum protected in the intraperitoneal test, whereas as much as 0.1 ml of a 1/4 dilution was required in the intracerebral test. The low *Ai/Ad* ratio, therefore, is at least partly explained by the high *Ad* value. The problem is thus reduced to an explanation of the relative inefficiency of equine encephalomyelitis antiserum in the direct intracerebral test.

This problem has been discussed in the literature, and the plausible explanation has been advanced that in the intracerebral test the proximity of the brain interferes with the reaction between virus and antibody. For reasons which will become apparent below, this explanation is unacceptable. Our theory follows a different line of reasoning.

Schaeffer and Muckenfuss (47, 48) and Friedemann, Zuger and Hollander (14) found independently that intracerebrally injected fluid always reaches the intraventricular fluid. Even after injection of colored fluid directly into the exposed cerebral cortex, the substance of the brain remains uncolored. The fluid either reaches the ventricles or runs out of the channel of injection into the subarachnoid space. In the direct test, therefore, the reaction between virus and antibody can take place only in the cell-free cerebrospinal fluid. It is tantamount to an *in vitro* reaction.

In the last analysis, therefore, the difference in the *Ai/Ad* ratios for the viruses of rabies and equine encephalomyelitis must be due to some fundamental difference in the mechanism of the virus-antibody reaction *in vitro*. It looks as if the virus of equine encephalomyelitis reacts very little, if at all, with its antibody in the cell-free spinal fluid, while the contrary holds true for the virus of rabies. To test the validity of this hypothesis, direct intracerebral tests were performed with incubated and non-incubated mixtures (20). The results of these experiments are recorded in tables 16 and 17.

As may be seen from table 16, the protecting dilution of antiserum in the non-incubated mixtures of rabies virus was 1/16 but in the incubated mixtures it was at least 1/8142. The potency, therefore, was at least 500 times higher in the incubated than in the non-incubated mixtures. This experiment shows clearly that rabies virus and its antibody react with each other *in vitro*.

On the other hand table 17 shows that the potency of equine encephalomyelitis antiserum is not at all increased by incubation. Since the serum was found to be so potent in the intraperitoneal test, it is clear that the virus of equine encephalomyelitis does not react with its antibody *in vitro* unless the latter is diluted to less than 1/2.

This fundamental difference between the two viruses is most interesting, but apparently it is no great help in understanding the mechanism of immunity in equine encephalomyelitis. On the contrary, it poses a most difficult problem. If equine encephalomyelitis virus does not react with its antibody *in vitro*, how is it possible that the serum protects passively against the intracerebral injection of the virus, and why is the *Ai/Ad* ratio exceptionally low?

The following considerations will show that the mechanism of the virus-antibody reaction in the indirect test must be different from that in the direct test.

The protecting dose in the direct test was 0.05 ml. If the mechanism of the reaction were the same in the direct and the indirect tests and if, consequently, the Ai/Ad ratio were exclusively determined by the distribution of the antibody

TABLE 16

Direct intracerebral tests with rabies virus in incubated and non-incubated mixtures

0.1 ml of a 1/100,000 dilution (100 MLD) of rabies virus was mixed with serial dilution of antiserum. In one series the mixtures were kept at room temperature for 24 hours. In another series, virus and antiserum were kept separately at room temperature for 24 hours and mixed immediately before injection. Mixtures were injected intracerebrally into guinea pigs weighing 200 g.

SERUM DILUTION	SURVIVAL* FROM INCUBATED MIXTURES	SURVIVAL* FROM NON-INCUBATED MIXTURES
1/16	S,S	S,S
1/32	S,S	S,11
1/64	S,S	S,10
1/128	S,S	S,9
1/256	S,S	9,9
1/512	S,S	9,9
1/1024	S,S	—
1/2048	S,S	—
1/4096	S,S	—
1/8192	S,S	—

* S = survived; numerals indicate day of death.

TABLE 17

Direct intracerebral tests with the virus of equine encephalomyelitis in incubated and non-incubated mixtures

0.1 ml of equine encephalomyelitis virus in a dilution of 1/640 (100 MLD) was mixed with serial dilutions of antiserum. As in table 16, the experiment was carried out with incubated and non-incubated mixtures in guinea pigs weighing 200 g.

ANTISERUM	SURVIVALS* FROM INCUBATED MIXTURES	SURVIVALS* FROM NON-INCUBATED MIXTURES
1/2	S,S	S,S
1/4	5,8	S,5
1/8	5,5	S,6
1/16	6,6	6,5
1/32	5,4	6,4
1/64	6,5	4,5
1/128	5,8	6,4

* S = survived; numerals indicate day of death.

between blood and brain, the ratio would be the same as in the experiments with diphtheria toxin, namely 6200:1, and Ai would have a value of 320 ml. Experimentally, however, an Ai value of 0.25 ml was found. If we consider that in the direct test the virus-antibody reaction takes place in the cell-free spinal

fluid, while in the indirect test virus and antibody react with each other in the substance of the brain, we can hardly escape the conclusion that in the indirect test the tissue cells provide something that is necessary for the reaction between virus and antibody.

Fortunately this is not an arbitrary hypothesis but fits in very well with the investigations of Sabin (46) on virus immunity. Both Andrewes (1) and Sabin have shown that the viruses of vaccinia, pseudorabies, and virus B do not react with their respective sera *in vitro* and consequently, resemble in this respect, the virus of equine encephalomyelitis. Sabin, however, found in carefully planned experiments that the above mentioned viruses are acted upon by their antibodies in the presence of susceptible tissue cells. He treated these cells first with antiserum, washed them and then added the virus. This procedure prevented the development of inclusion bodies which formed in cells treated with the virus alone. Sabin assumes that the antibody coats the surface of the cells and thus prevents the viruses from entering them.

Similar experiments with the virus of equine encephalomyelitis have not been performed. It appears, however, very plausible to assume that the discrepancy between the inefficiency of the encephalomyelitis antiserum *in vitro* and its high efficiency in the animal body is explicable along similar lines as in the experiments with vaccinia virus, pseudorabies, and virus B.

An experimental analysis of the Ai/Ad ratios of the viruses of rabies and equine encephalomyelitis has thus revealed the existence of two groups of neurotropic viruses with entirely different mechanisms of immunity.

It will be of great interest to determine the distribution of other neurotropic viruses between these two groups. Although, thus far, this problem has not been approached systematically, observations reported in the literature give some hints. It has already been mentioned that the pseudorabies virus and B virus fail to combine with their antibodies *in vitro* and behave in this respect like equine encephalomyelitis virus. More recently Lennette and Koprowski (35) have shown that the result obtained by Olitsky and Harford (42) for the virus of equine encephalomyelitis holds true for a number of other viruses. In experiments with the viruses of Eastern and Western equine encephalomyelitis, Venezuelan encephalomyelitis, Western Nile disease, St. Louis encephalomyelitis, Japanese encephalomyelitis and yellow fever, it was found that antibodies can be easily identified in the intraperitoneal test in young mice, while this was difficult or impossible in the intracerebral test. It would appear that as far as the mechanism of immunity is concerned, all these viruses belong in one group. It must be emphasized, however, that some of these viruses are closely related to each other.

On the other hand, the virus of poliomyelitis seems to resemble the virus of rabies. Olitsky and Cox (41), Harmon and Harkins (29), Gordon (28), and Schultz and Gebhardt (50) found difficulties in protecting rhesus monkeys passively against the intracerebral injection of poliomyelitis virus even by the intravenous administration of the largest amounts of a highly potent immune serum. Since antibodies against the virus of poliomyelitis can be identified with

the aid of the direct intracerebral test in monkeys, the findings of the above mentioned authors seem to indicate that the *Ai/Ad* ratio for the virus of poliomyelitis is very high and resembles in this respect the virus of rabies.

It would be premature to predict which type of virus is more frequently met. Much more experimental material will be required to decide this question. It need hardly be emphasized that our knowledge in this field is still very incomplete. To characterize a neurotropic virus, it will be necessary to determine the following data: the *Ai/Ad* ratio, the *Ad* value in the intracerebral test for incubated and non-incubated mixtures, determination of the antibody titer in the intracerebral test and the intraperitoneal test in young mice, and if technically possible, the decision of the question whether the antibody requires the cooperation of tissue cells. Only when these questions can be answered for a sufficient number of neurotropic viruses, will it be possible to develop a well founded theory of immunity in neurotropic virus diseases. The results reported in this review, however, indicate the trend along which further work is desirable.

It has been seen in the preceding paragraphs that the mechanism of the virus-antibody reaction determines to a large extent the *Ad* value and therefore, indirectly the *Ai/Ad* ratio. The question arises, whether, in addition, the mechanism of the virus-antibody reaction also influences the *Ai* value. On the basis of the existing experimental evidence, it is impossible to rule out this possibility. It would even be plausible to assume that an antibody which coats the cells might be more effective than an antibody which neutralizes the virus outside the cells. Perhaps certain observations on passive immunity might become understandable along these lines. We have mentioned how difficult it is to protect experimental animals passively against the intracerebral injection of the viruses of rabies and poliomyelitis. On the other hand, the experiments reported in this review as well as the investigations of Cox and Olitsky (4), Howitt (30), Zichis and Shaughnessy (55), and Olitsky *et al.* (43) show that passive protection is obtained very easily with the virus of equine encephalomyelitis. Although the interpretation of these observations certainly calls for further experimental work, these considerations may be mentioned in order to show that further studies on immunity reactions *in vivo* and particularly on the *Ai/Ad* ratio might lead to new viewpoints in the field of virus immunity.

Permeability of the cerebral capillaries to antibodies

For many years it has been the prevailing opinion that the cerebral capillaries are impermeable to antibodies. This concept is at variance with our results obtained in the indirect intracerebral tests with tetanus toxin and the virus of equine encephalomyelitis. The question of the permeability of the cerebral capillaries to antibodies, therefore, called for a special investigation.

The concept of the impermeability of the so called blood-brain barrier to antibodies was based on the very low concentration of antibodies in the cerebrospinal fluid. Since according to the older theories, antibodies were supposed to reach the central nervous system only by way of the choroid plexus, the almost complete absence of antibodies from the spinal fluid was considered as evidence that

antibodies do not reach the central nervous system at all. The difficulty in immunizing experimental animals passively against the virus of rabies or poliomyelitis was explained by some authors on this basis.

This argument is no longer significant, for it is now the consensus of opinion that the exchange of substances between blood and brain takes place directly through the walls of the cerebral capillaries (11, 52, 10). There are, however, other observations on record which were interpreted as indicating that at least in some species the cerebral capillaries are impermeable to antibodies. This concept goes back to old experiments of Roux and Borrel (45). They found that rabbits actively or passively immunized against tetanus toxin were not protected against the intracerebral injection of a single lethal dose of the toxin. Later these experiments were repeated by Descombey (5), and Mutermilch and Salamon (40) in guinea pigs with entirely different results. Guinea pigs immunized in the same way as rabbits withstood the intracerebral injection of as much as 20 lethal doses of tetanus toxin. From these results the French authors concluded that the cerebral capillaries are permeable to antibodies in the guinea pig but impermeable to them in the rabbit.

This explanation appears rather artificial. The experiments reported in this review suggested an entirely different explanation. In the first place it has been shown that the Ai value depends on the size of the experimental animal. For this reason alone the protecting dose of antitoxin in the indirect intracerebral test should be 10 times higher in the rabbit than in the guinea pig. To make results in the two species comparable, therefore, the Ai value should be divided by the plasma volume, P.

In the second place the Ai value, in contradistinction to the Ai/Ad ratio is dependent on the combining power of the test dose of toxin. This is of paramount importance if, as in the experiments of the French authors, the test dose is measured in terms of lethal doses. Since the guinea pig is much more susceptible to tetanus toxin than the rabbit, one lethal dose in the rabbit represents a much higher combining power than one lethal dose in the guinea pig and requires for its neutralization a much higher amount of antitoxin.

The correctness of these considerations could be demonstrated in a convincing manner by experiments with diphtheria toxin (14). The intracerebral lethal dose of diphtheria toxin, in contrast to tetanus toxin, is the same for the rabbit and the guinea pig. If Ai/P were actually determined by the combining power of the test dose, it should , therefore, have the same value in both animals. On the other hand, if the cerebral capillaries in the rabbit were impermeable to diphtheria antitoxin, the experiments with diphtheria toxin would be a replica of those with tetanus toxin. The experiment gave a clear cut answer to this question. As will be seen from table 18, in the experiment with diphtheria toxin, the Ai/P ratio had substantially the same value in the guinea pig and the rabbit, whereas in the experiment with tetanus toxin, the Ai/P value was 200 times higher in the rabbit than in the guinea pig.

There is another way to show that the cerebral capillaries are equally permeable to antitoxins in the rabbit and the guinea pig. If Ai/P is determined exclusively

by the combining power of the test dose of toxin it should have the same value in the rabbit and the guinea pig if, irrespective of the lethal dose, equal amounts of toxins are given to both animals. The experiment recorded in table 19 (14) shows that the Ai/P values, although not identical, approach each other closely. A complete agreement between theory and experiment can hardly be expected since as was shown previously the Ai values for tetanus toxins in the guinea pig

TABLE 18

Determination of Ai for 10 lethal intracerebral doses of tetanus and diphtheria toxins in the rabbit and the guinea pig

10 lethal doses of tetanus toxin 388, 0.25 ml for the rabbit, and 0.0025 ml for the guinea pig; 10 lethal doses of diphtheria toxin, 0.0025 ml for both the rabbit and the guinea pig.

ANIMAL	TETANUS ANTITOXIN (ml)	SURVIVALS*	DIPHTHERIA ANTITOXIN (ml)	SURVIVALS*
Rabbit (2500 g)	2	S,1	3	S,S
	1	1,5	2	S,6
	0.5	2	1	4,4
			0.5	5,6
Guinea pig (250 g)	0.001	S	0.5	S,S
	.0005	9	0.25	S,7
	.00025	10	0.125	5,6
	.0002	3,3		
	.0001	3.6		

* S = survived; numerals indicate day of death.

TABLE 19

Experiments with tetanus toxins A and C

The test dose was the same in the guinea pig and the rabbit (10 lethal rabbit doses). The Ai values were determined in the usual way.

TOXIN	TEST DOSE OF TOXIN (ML)	Ai/P* (RABBIT)	Ai/P* (GUINEA PIG)
A	0.025	0.02	0.03
C	0.00625	0.02	0.05

* P = plasma volume

are not exclusively determined by the combining power of the test dose but also by the avidities of the individual toxins for nerve tissue.

The clarification of this problem is instructive because it shows how dangerous it is to compare experiments in different animal species on the basis of the Ai values alone.

SUMMARY

The investigations reviewed in this survey deal with a hitherto rather unexplored field of immunology. The classical method of evaluating immune sera

consists of mixing pathogenic agent and immune serum in the test tube and injecting the mixture into the experimental animal. Under these conditions the reaction between the two components takes place almost exclusively outside the animal body and the experimental animal serves only as an indicator of the unneutralized pathogenic agent. These reactions are in reality *in vitro* reactions. When immune serum is given for therapeutic or prophylactic purposes, however, the reaction between pathogenic agent and antibody takes place exclusively within the animal body. It is these immunity reactions *in vivo* with which the present article deals.

It was the final goal of these investigations to elaborate or at least to lay the foundation for a rational dosage of immune sera. For this purpose an attempt was made to determine in the animal experiment the ratio between the therapeutic dose of immune sera and their neutralizing dose *in vitro*, and to investigate the mechanism which determines this ratio.

It has been known since the early days of immunology that the therapeutic dose is usually infinitely larger than the neutralizing dose but the reason for this difference was imperfectly understood. It was realized early in the course of these investigations that this difference is largely due to the fact that in the diseased human body the pathogenic agent is within the tissue while the antibody circulates in the blood separated from the pathogenic agent by the capillary wall. To simulate these conditions as closely as possible, serial dilutions of immune serum were injected intravenously while immediately afterwards a constant dose of the pathogenic agent was injected into some tissue (skin, brain or muscle). Experiments of this type were designated as indirect tests and the minimal neutralizing dose of immune serum as Ai. With some qualifications, Ai may be considered as representing the therapeutic dose.

The neutralizing dose of immune serum was determined by mixing serial dilutions of immune serum and a constant dose of pathogenic agent *in vitro* and injecting 0.1 ml of each mixture into the same tissue as in the indirect test. These experiments were designated as direct tests and the neutralizing dose of immune serum as Ad.

The ratio, Ai/Ad, between the therapeutic and the neutralizing doses has been determined for a number of pathogenic agents. It has been shown that the mechanism determining the Ai/Ad ratio in the case of diphtheria toxin is exclusively determined by the distribution of antitoxin between blood and tissue and can be expressed by the formula $Ai/Ad = KP/V$, where K is the coefficient of distribution of antitoxin between blood plasma and tissue, P the plasma volume, and V the volume of fluid injected into the tissue. In skin test experiments on rabbits weighing 2500 g, Ai/Ad was found to have a value of 22,000. In a child weighing 30 Kg, the ratio would be approximately 440,000. This figure gives an approximate idea of the ratio between the therapeutic and the neutralizing doses of antitoxin in the case of diphtheria. Actually this is a minimum value. As is well known the therapeutic dose increases with the interval between the onset of the disease and the administration of antitoxin.

According to the above distribution equation, the Ai/Ad ratio should be the

same for all pathogenic agents; but in the case of other pathogenic agents, conditions are not so simple. Pathogenic agent and antibody may react not only with each other but also with the tissue and accordingly the ratio may be very different from those found for diphtheria toxin. Particularly interesting and unexpected observations were made in the case of tetanus toxin. The *Ai/Ad* values were determined for a considerable number of tetanus toxins in indirect intracerebral and intramuscular tests. Not only were the results very different from those obtained with diphtheria toxin but the *Ai/Ad* ratios for different tetanus toxins differed among themselves very considerably. For some toxins the ratios were 100 times higher than for others. Experimental analysis of these observations showed that the *Ai/Ad* values were determined by the avidities of the various toxins for nerve tissue.

Extreme differences in *Ai/Ad* were found in experiments with neurotropic viruses. While the ratio was at least 1280 for the virus of rabies, it had the very low value of 5 for the virus of equine encephalomyelitis. A further experimental analysis of these results uncovered a fundamental difference in the mechanism of immunity in the case of these two viruses. While the virus of rabies readily combines with its antibody *in vitro* even when the immune serum is highly diluted, the virus of equine encephalomyelitis does not react with its antibody *in vitro* unless the antiserum is undiluted. Since in the direct test virus and antibody react with each other exclusively in the cell-free ventricular fluid, the neutralizing dose in the direct test must be very high for the virus of equine encephalomyelitis. The fact that the *Ai* value is not proportionally high shows that the mechanisms of immunity in the direct and the indirect tests must be different. In analogy with the investigations of Sabin on the viruses of vaccinia and pseudorabies, and on virus B, it has been assumed that the virus of equine encephalomyelitis is acted upon by its antibody only when the latter has been fixed by nerve cells.

In view of the complicated nature of immunity in neurotropic viruses it can hardly be predicted to what extent our results can be generalized for other neurotropic viruses. Further investigations are called for to determine whether there exists any general rule correlating the *Ai/Ad* ratios of viruses with their ability or inability to react with their antibodies *in vitro*. There are some indications in our experiments that active or passive immunization against the intracerebral injection of viruses may be easy if the antibody is fixed by nerve cells while it may be difficult if the antibody reacts with the virus directly. This question also will require more extensive investigation.

The skin tests with diphtheria toxin, and the intracerebral tests with tetanus toxin and neurotropic viruses, have in common that the reaction to the pathogenic agent is observed at the site of its injection. Indirect tests, however, can be carried out in such a way that the reaction to the pathogenic agent is observed in organs remote from the site of injection. A case in point is the indirect intramuscular test with diphtheria toxin where the dose of antitoxin was determined which protects the experimental animals against death. In the direct test toxin and antitoxin were injected intravenously. In these experiments the antitoxin requirements were approximately the same for intramuscularly and intravenously

injected toxin. This is due to the fact that diphtheria toxin reaches the heart and the adrenals by way of the circulation. It is irrelevant, therefore, whether the toxin is neutralized at the site of its injection or in the blood stream.

Analogous experiments with tetanus toxin gave an entirely different result. Intramuscularly injected toxin required for its neutralization up to 80 times more antitoxin than intravenously injected toxin. This is due to the fact that tetanus toxin reaches the central nervous system by way of the peripheral nerves. It is essential, therefore, that the intramuscularly injected toxin be neutralized at the site of its injection.

Finally it has been shown that the method of the indirect test can be used in studies on capillary permeability. For this purpose the substance under investigation is mixed with the test dose of toxin in the indirect test while the antitoxin is given intravenously as usual. An increase in capillary permeability is indicated by the enhanced neutralizing effect of the antitoxin. With the aid of this convenient method, the effect of a large number of substances has been studied. It is remarkable that most of the substances which affect the capillaries at all, increase their permeability to such an extent that the concentration of antitoxin on both sides of the capillary wall becomes identical.

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